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Determining the sensitive developmental stages of intersex induction in medaka (*Oryzias latipes*) exposed to 17β-estradiol or testosterone

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Abstract

Certain environmentally persistent compounds can adversely affect reproduction by acting as steroid hormone agonists or antagonists. The goal of the present study was to determine the developmental stage most susceptible to exogenous hormone (estradiol and testosterone) exposure using a small teleost model. In the first (pilot study) of two experiments, medaka (*Oryzias latipes*), at varying developmental stages, were bath-exposed to 5 μg/l 17β-estradiol for 24 h. At 5 months of age, fecundity, fertility and embryo and larval viability (reproductive success) were investigated in control and exposed groups. Fish at 1, 1.5, 2 and 5.5 months of age were also sampled, processed and examined histologically for gonadal alteration. No significant differences in mortality, gonadal morphology, body weight, sex-ratio or time to maturity were seen between control and exposed fish. At 5 months, however, when exposure groups were compared to controls, significant differences were seen in reproductive success and viability of offspring. A second experiment exposed embryo stage 10, and 1-, 7- and 21-day-old larvae for 6 days to 15 μg/l 17β-estradiol or 100 μg/l testosterone. No significant differences were seen at 5 months in mortality, body weight, or time to sexual maturity. However, sex-ratios were significantly biased toward female in the stage 10, 1- and 7-day post-hatch estradiol exposure groups. No significant changes in sex-ratio were associated with testosterone exposure at any developmental stage. Further, intersex gonads were observed in fish from all groups exposed to 15 μg/l estradiol. Only those fish exposed as newly hatched fry or at 1 week post-hatch displayed intersex gonads following 100 μg/l testosterone exposure. Data from these experiments show that newly hatched fry are that life stage most sensitive to hormone exposure and the most appropriate to use in determining effects of known endocrine-disrupting compounds.

Keywords: Intersex; Induction; 17β-Estradiol; Medaka; *Oryzias latipes*

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Certain environmentally persistent compounds have been shown to alter (i.e. disrupt) the endocrine system in laboratory and wild fishes (Gray & Metcalfe, 1997; Jobling, Sheahan, Osborne, Matthiessen & Sumpter, 1996). The question remains: do environmentally relevant concentrations of these xenobiotics affect individual reproduction and thereby potentially impact populations? Our goal is to develop a short-term in vivo model and apply it to answer this question. Not only is exposure concentration important, but also that life stage at which exposure occurs, i.e. the window of susceptibility. Although not shown in fishes, studies of reptilian species suggest developmental exposure will most likely have a more profound effect than at any other life stage (Guillette, Crain, Rooney & Pickford, 1995).

Fish in general, and medaka (*Oryzias latipes*) in particular, have been proposed as models for detecting adverse effects of chemicals on reproduction (Arcand-Hoy & Benson, 1998; Koger, Teh & Hinton, 1999). More prolific than other egg-laying aquarium fishes (Myers, 1952), medaka have been functionally sex-reversed in both directions (male to female, female to male) via dietary hormone exposure (Yamamoto, 1961). Other than rare (unpublished observations in this laboratory; see also Hartley, Thiyagarajah & Mizell, 1995) ectopic hermaphroditic germ cells in the cranial cavity, no cases of spontaneous intersex have been reported in medaka.

Experiment I

The exposure regime employed for this pilot study followed that used by Hartley, Thiyagarajah, Anderson, Broxson, Major and Zell (1998) which showed intersex induction following 48-h bath-exposure of medaka fry to estradiol examined at 2 weeks post hatch (Hartley et al., 1998). Here, we used medaka collected and divided into exposure groups, including: stage 10 (early high blastula; Kirchen & West, 1973), hatch, day 21 post hatch and unexposed (control). All fish were bath-exposed to 5 μg/l 17β-estradiol for 24 h (three replicates, 30 fish per replicate). Stage 0 exposure was done by bath-exposing actively breeding adults to 5 μg/l estradiol, and collecting the resultant embryos. Again, three replicates (30 fish per replicate) were collected. After exposure, all fish were raised in clean water and at 5 months of age were examined. No significant differences were seen in mortality, gonadal morphology, body weight, or time to maturity between control and exposed fish. Regardless of stage of exposure, significant reduction in reproductive success (fecundity, fertility and embryo/larval viability) was seen in all exposure groups. Sex ratio, while not significantly different, was biased toward females in the newly hatched exposure group (pilot study data not shown). To examine histologic effects of hormone exposure, concentration and time of exposure were increased (see below). In addition, effects of androgen exposure were determined using testosterone.

Experiment II

Medaka (three replicates, 30 fish per replicate) were bath-exposed (daily static-renewal) to 15 μg/l 17β-estradiol, 100 μg/l testosterone or bath only (controls) for 6

days. Exposures were initiated at the following developmental stages: stage 10, hatch, day 7 and day 21 after hatch. When fish reached 5 months of age, sex-ratio and gonadal histology were investigated. Also, fecundity, fertility and embryo and larval viability were determined using breeding groups from each exposure stage (three replicates, two females and two males per replicate). No significant differences in mortality, body weight or time to maturity were seen between control and exposed fish. However, reproductive and histological changes were associated with exposure (see below).

Gonadal morphology

Regardless of developmental stage at initiation of exposure to 15 μg/l estradiol, individuals with intersex (presence of immature oocytes within the testis) gonadal morphology were seen and no intersex gonads were observed in control fish. Fish from the newly hatched fry and day 7 testosterone exposure groups also displayed intersex gonadal morphology. Compared to estradiol-induced intersex gonads, which showed either few immature oocytes in an apparently functional testis (Fig. 1) or several immature oocytes within the testicular duct along with mature spermatozoa (Fig. 2a, b), testosterone exposed fish had a more pronounced intersex morphology. One intersex fish exposed to testosterone day 1 post hatch produced both mature ova and mature sperm (Fig. 3). The duct in this animal is under analysis to determine if this fish was a true hermaphrodite.

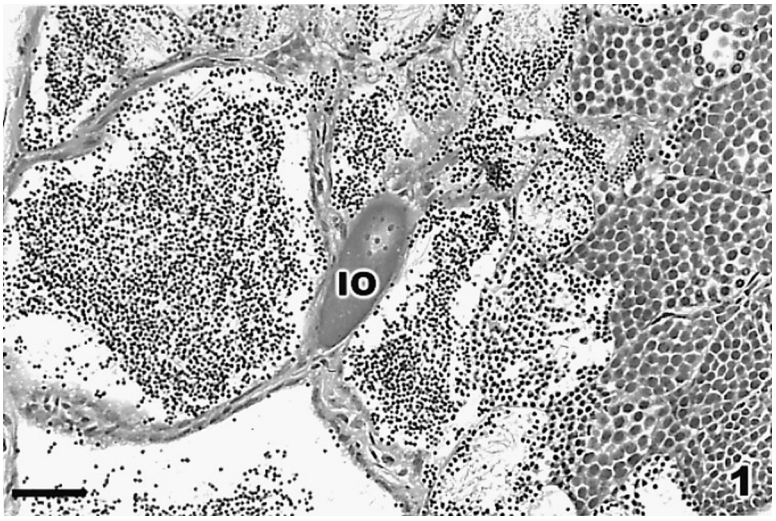

Fig. 1. Testis of a newly hatched fry exposed to 15 μg/l estradiol showing one immature oocyte (IO) despite a functional morphology characterized by the presence of mature, flagellated spermatozoa. Scale bar = 22 μm.

Fertility, embryo/larval viability

As evidenced by a higher percentage of unfertilized eggs collected from groups exposed to either hormone, fertility was apparently altered. However, the number of oviposited eggs (fertilized and unfertilized) collected was not significantly different. Based upon number of embryos that successfully hatched and the resultant larvae

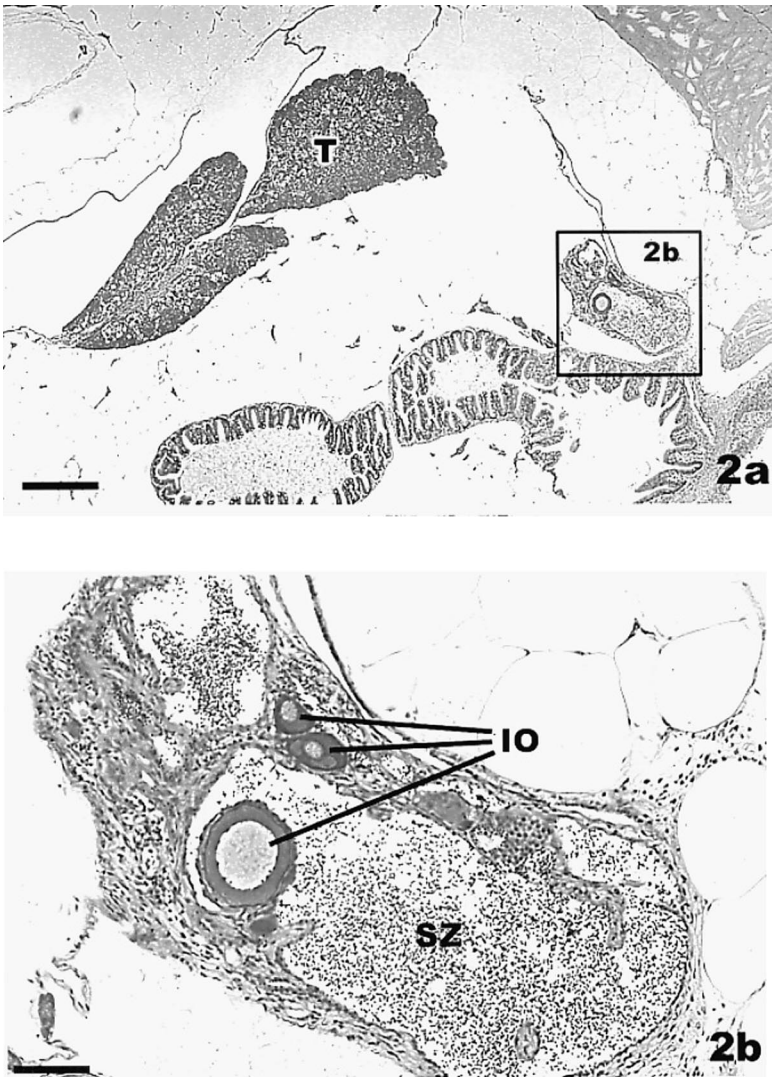

Fig. 2. (a) Gonad of a newly hatched fry exposed to 15 μg/l estradiol with normal testis morphology (T). However, several immature oocytes are present within the testicular duct system (insert 2b). Scale bar = 218 μm. (b) Immature oocytes (IO) are shown within the duct system of a medaka fry exposed to 15 μg/l estradiol, along with mature spermatozoa (SZ). Scale bar = 44 μm.

that survived to one week post-hatch, embryo/larval viability was significantly decreased in all exposure groups (data not shown).

Sex ratio

Stage 10, hatch, and day 7 estradiol exposure groups had significantly biased sex ratios: 15% male/85% female, 13% male/87% female and 24% male/76% female, respectively. There was no significant difference in sex ratio compared to control ratios for any developmental group exposed to testosterone. Sex ratios were determined by secondary fin characteristics and not by gonadal morphology.

Histologic evaluation

Fish were euthanised with a lethal concentration of MS-222 (100 mg/l), and their body cavities surgically exposed to ensure optimum fixation (10% buffered formalin). After 24 hours, fish were transferred to Bouin's fixative for another 24 h to decalcify bones. Following graded ethanol dehydration, all samples were embedded in either paraffin or glycol methacrylate (GMA), serially sectioned (4–4.5 μm) and stained with hematoxylin and eosin before viewing under a light microscope.

In experiment II, estradiol caused intersex in all exposure groups, while intersex gonadal morphology was only observed in day 1 and day 7 post hatch testosterone exposure groups. This suggests that males feminized by estradiol exposure still had bipotent germ cells present in the testis up to 21 days post hatch, while female fish masculinized by testosterone had sufficient differentiation of germ cells by day 21 to alleviate the effects of androgen exposure. Hence, no intersex was observed following testosterone exposure initiated at day 21 post hatch. Data from both the 24-h

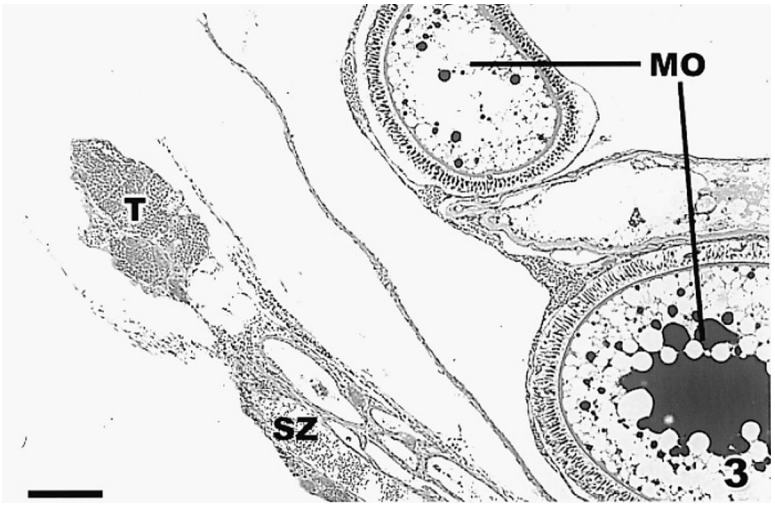

Fig. 3. Newly hatched fry exposed to 100 μg/l testosterone showing both mature oocytes (MO) and mature spermatozoa (SZ). Scale bar = 88 μm.

and 6-day exposure studies suggest that newly hatched fry, rather than intact chorionated embryos, are the most sensitive developmental stage to estradiol or testosterone exposure. Further, while reproductive endpoints such as fertility and larval viability need to be incorporated into a study of endocrine disruption, careful histological evaluation using high-resolution light microscopy should also be included in post-exposure analysis, especially if exposure occurs during sensitive life stages.

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